Nondestructive Biomarkers in Ecotoxicology

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The aim of this article is to attempt a concise review of the state of the art of the nondestructive biomarkers approach in vertebrates, establishing a consensus on the most useful and sensitive nondestructive biomarker techniques, and proposing research priorities for the development and validation of this promising methodology. The following topics are discussed: the advantages of the use of nondestructive strategies in biomonitoring programs and the research fields in which nondestructive biomarkers can be applied; the biological materials suitable for nondestructive biomarkers and residue analysis in vertebrates; which biomarkers lend themselves to noninvasive techniques; and the validation and implementation strategy of the nondestructive biomarker approach. Examples of applications of this methodology in the hazard assessment of endangered species are also presented. — Environ Health Perspect 102(Suppl 12):49–54 (1994)

Key words: biomarkers, nondestructive techniques, endangered species, marine mammals, mixed-function oxidases, esterases

Biomarkers: Destructive and Nondestructive Use

In the last 20 years, ecotoxicology has been increasingly concerned with the use of biomarkers to evaluate the biologic hazard of toxic chemicals and in the assessment of environmental health. A biomarker is defined by the National Academy of Sciences (*1*) as "a xenobiotically-induced variation in cellular or biochemical components, processes, structures, or functions that are measurable in a biological system or samples." Such "variations" can indicate the magnitude of the organism's response to contaminants as well as provide the causal link between the presence of a chemical and an ecologic effect. The concept of biomarkers in the evaluation of environmental risk has captured the attention of regulatory agencies and is currently being assessed by several research commissions. This interest is confirmed by the increasing number of specialist manuals (*2–6*).

The central feature of this methodological approach is to "quantify exposure and its potential impact by monitoring biological end points (biomarkers) in feral animals and plants as indicators of exposure to and the effects of environmental contaminants" (*5*). However, in environmental contamination problems, the terms of investigation may shift from evaluation of environmental health using sentinel species as bioindicators to a more specific investigation of the "health" of a population or an endangered species in a situation of already ascertained environmental pollution. This inversion of terms inevitably leads to a demand for analytical and sampling methods that are compatible with the protection and conservation of the organism to be studied. In light of this increasingly important requirement, it is essential to focus on the use of nondestructive biomarkers.

The choice of nondestructive biomarkers over destructive biomarkers is not only an ethical one. The author does not wholly agree with the ideology of certain radical environmental movements for which the animal organism, as an individual, must be saved at all costs. From the ecologic point of view, the value of a population or a community is greater than that of an individual. With this in mind, the loss of a few individuals for research purposes is permissible if the data obtained contributes to the conservation of the population or community studied. On the other hand, there is the problem of the "ethic of the researcher." One may often ask whether the researcher is more harmful to the population than the contaminants studied. Several examples exist of "case studies" in which populations of protected species, already heavily stressed by anthropogenic disturbance and contaminants, have been further reduced in number by "wildcat" sampling on the part of short-sighted ecotoxicologists. Apart from ethical considerations, destructive testing in vertebrates may be unacceptable under many conditions, for example, in the hazard assessment of protected or threatened species, or when the number of animals available at a site is limited, or when sequential samples from the same individual are required for time-course studies.

The use of noninvasive methods of monitoring the health of species and populations at risk has infrequently been the subject of investigation by the "biomarker scientific community" (*6–10*). The concept of nondestructive testing has great merit and potential, but progress in its development and application to environmental health assessment is hampered because no one had brought all the different methodologies and concepts of nondestructive testing together in an organized way until the recent book edited by Fossi and Leonzio (*6*).

The aim of this article is to attempt a concise review of the state of the art, to establish a consensus on the most useful and sensitive nondestructive biomarkers, and to propose research priorities for the development and validation of this promising methodology. The limited amount of information available on this methodologic approach makes it important to clarify certain important aspects. For example, the advantages of the use of nondestructive strategies in biomonitoring programs and the research fields in which nondestructive biomarkers can or should be applied; the biologic materials suitable for nondestructive biomarkers and residue analysis; which biomarkers lend themselves to noninvasive techniques; the validation and implementation strategy of the nondestructive biomarker approach.

The Department of Environmental Biology of Siena University has recently been concerned with development and validation of the nondestructive biomarkers approach in experimental and field studies.

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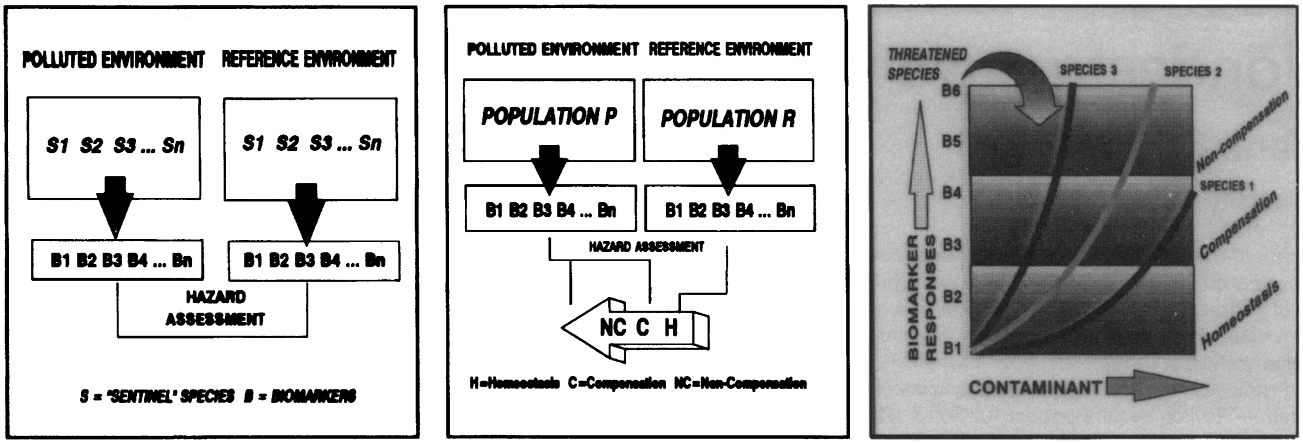

A **B** **C**

Figure 1. Three applications of nondestructive biomarkers in biomonitoring programs: (*A*) environmental hazard assessment; (*B*) population hazard assessment; (*C*) identification of "species at risk."

Its application to hazard assessment of endangered species of high vertebrates (e.g., marine mammals), and the development models of the relationship between nondestructive and destructive biomarkers in several vertebrate species, are two crucial aspects of present and future research. This article reviews some of these recent experimental results.

Nondestructive Biomarkers in Biomonitoring Programs

First, it is important to distinguish advantages of nondestructive methods over conventional invasive or destructive techniques. In this section we discuss the specific cases of biomonitoring in which nondestructive biomarkers can or must replace destructive techniques.

Environmental Hazard Assessment

Nondestructive biomarkers can replace destructive techniques in environmental monitoring (Figure 1*A*). With the aim of evaluating the "health" of a given environment (terrestrial, marine, or freshwater), a series of sentinel species can be tested with a suite of nondestructive biomarkers and the values obtained compared with results from the same species in a reference area. However, it is important to recognize that limitations in our current understanding of the molecular and biochemical mechanisms of toxic action add to the presence of confounding variables (such as seasonal changes, temperature, nutritional factors, sex, age, etc.) that may prevent unequivocal interpretation of biomarker responses, especially in relating them to a specific consequence at high levels of biological organization (*2*).

This procedure has hitherto been used with destructive sampling in aquatic and terrestrial environments, based on the analysis of target tissues such as liver, kidney, and brain. The use of noninvasive techniques in environmental monitoring would, however, have many important advantages (*6*): population decrements are avoided and legislative restrictions on the sacrifice of higher vertebrates (reptiles, birds, and mammals) can be overcome; ecologically important species having reduced numbers can be analyzed. Such species could not be tested using invasive methods without further endangering the population; a larger number of individuals can be sampled per station, which gives the data greater statistical weight; if animals can be recaptured, time series of measurements of the same biomarkers can be obtained from a given individual subjected to constant or variable chemical insult. The toxicological data thus acquired could otherwise only be obtained in laboratory experiments; in laboratory studies, the role of endogenous (sexual cycle, age, nutritional status, etc.) and exogenous (temperature, daylight, etc.) factors in the variation of certain biochemical or physiologic (biomarker) responses can be studied in the same individual (thus excluding intra specific variation);

Hazard Assessment in Populations of Endangered Species

The principal ecotoxicologic application of nondestructive biomarkers is in the hazard assessment of endangered species of vertebrates (*6*). Incidents of drastic reductions in higher vertebrate populations (e.g., marine mammals, endangered bird species, etc.) linked to the presence of contaminants make it indispensable to develop techniques of biomonitoring and hazard assessment based on nondestructive methods. A good example of the application of the nondestructive strategy is the use of blood esterase assay in population of wild birds accidentally exposed to organophosphorus insecticides (*7,8,11*). This approach has allowed the nondestructive assessment of toxicologic risk.

In the risk assessment of an endangered population suspected to be exposed to toxic substances, nondestructive biomarkers may be applied in the following way. A series of nondestructive biomarkers may be tested in the population in question and compared with data from a reference population (Figure 1*B*). The difference in biomarker values in relation to the spectrum of homeostasis, compensation, and non compensation responses, gives a measure of the risk of the population studied. In this case, the goal of biomarker research is to identify how biomarker responses correspond to different levels of departure from normal homeostasis, as proposed by Depledge (*12*).

Identification of Species at Risk

Nondestructive biomarkers can be used to identify species potentially at risk in a polluted environment. This research is based on the assumption that interspecific differences in susceptibility to contaminants exist in any class of vertebrates. For example, it is well known that different species of wild birds have different levels of tolerance to lipo soluble xenobiotics (*13*). Such differences are mainly determined by the capacity of the mixed function oxidase detoxifying system, and are expressed as differences in adaptability or non adaptability to survive in a polluted environment.

In general terms, the vulnerability of a species to environmental contamination depends on two main factors, the possibility of exposure and the intrinsic sensitivity of the species. The first aspect can be checked by monitoring the habitat for the chemicals concerned. The biomarker approach may be used to assess intrinsic sensitivity (*5*). Biomarkers can tell us whether or not a species is responsive, and whether or not it transforms a certain chemical into a toxic metabolite. If our goal is to identify the species at risk in a particular polluted environment, the use of noninvasive techniques (mainly biomarker of effect) should be directed towards the evaluation of interspecific differences in response to the sum, known or unknown, of the polluting agents. With this aim, a suite of nondestructive biomarkers may be tested in several species of the same class suspected to be threatened (for example, fish-eating birds exposed to biomagnification through the marine food chain). The differences between biomarker values in different species, living in the same polluted environment, in relation to the spectrum of homeostasis, compensation and non-compensation, permits the identification of the species potentially at risk or threatened species (*6*) (Figure 1*C*).

An Example of Application: the Case of Marine Mammals

Noninvasive techniques have been successfully applied in the biomonitoring of populations of Mediterranean marine mammals. Chemical analysis (*14,15*) is performed and biochemical and cytochemical biomarkers were analyzed (*9,10*) in skin biopsy specimens. In a recent study (*10*), the use of a conventional biomarker, mixed-function oxidase activity, in a "nondestructive" way, was proposed. Benzo[*a*]pyrene monooxygenase activity (BPMO) was evaluated in skin biopsy specimens of free-ranging cetaceans. The specimens were obtained by a noninvasive method (dart) from striped dolphins (*Stenella coeruleoalba*) ($n = 7$) and fin whales (*Balaenoptera physalus*) ($n = 9$) in the northern Tyrrenian sea during the summer of 1991. Figure 2*A* shows the geometric mean values of BPMO activities in the skin samples and mean values of PCBs and DDTs. BPMO activity was four times higher in striped dolphins than in fin whales ($p < 0.020$). This difference in enzyme activity reflects the dramatically different levels of organochlorines in the subcutaneous blubber of the two species (*15*). In this study, the levels of PCBs and DDTs were 12 times ($p < 0.0005$) and 9 times ($p < 0.0005$) higher, respectively, in the striped dolphin than in the fin whale. The difference in organochlorine bioaccumulation in the two species is commonly related to the different positions in the marine food chain: the fin whale is a plankton feeder and the striped dolphin is a terminal consumer. Organochlorines are know to induce MFO activity in fish, bird and mammal liver. The main explanation of this interspecific difference in enzyme responses lies in the chlorinated hydrocarbon induction processes. The phenomena of MFO induction in mammal skin are well documented in rats after cutaneous administration of PCBs (*16*). Plotting the sum of organochlorines with BPMO activity, we find two species-specific sets of points showing an overall trend of increasing enzyme activity with increasing levels of contaminants (Figure 2*B*). The results of this preliminary study suggest that biomarkers together with the results of chemical residue analysis can be used in a combined approach for evaluating toxic risk to populations of marine mammals.

Biological Material Suitable for Nondestructive Biomarker Studies

The nondestructive studies can be divided into four categories (6): purely nondestructive methods, such as taking blood samples, after which the animal is released unharmed; invasive but nonlethal techniques, such as liver and muscle biopsies; techniques that can be performed without harm, such as hair and feather sampling. These materials are generally collected, but not only from animals that have died or been killed for some other reason. Even museum specimens can be used and greatly extend the temporal range of the study; studies on eggs which, while destructive to the egg, involve minimal harm to the species.

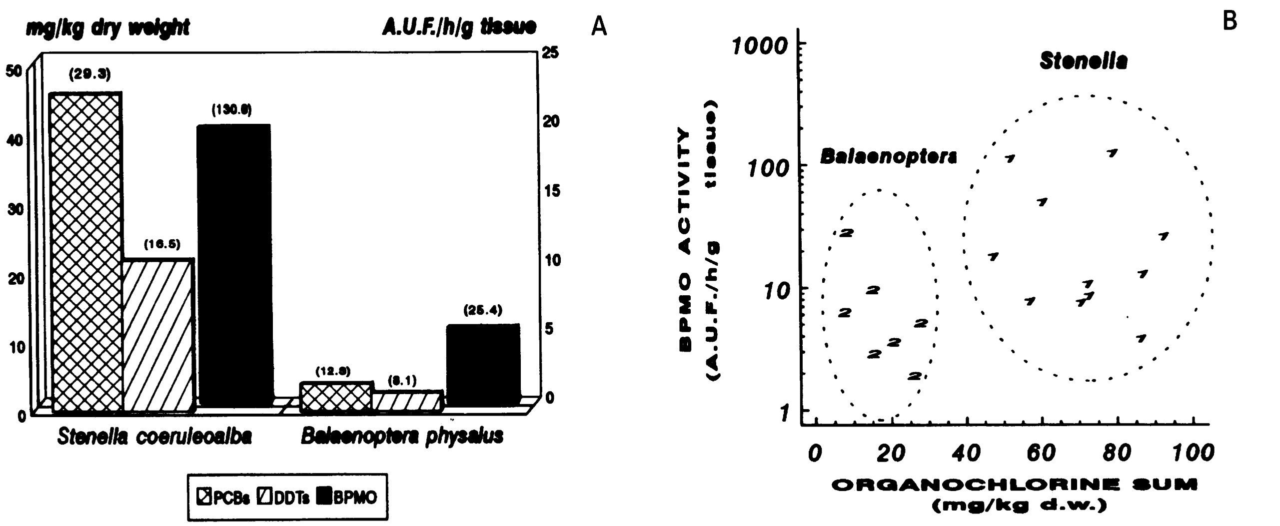

Figure 2. (*A*) BPMO activities and organochlorine levels in skin biopsy specimens of Mediterranean *Stenella coeruleoalba* ($n = 7$) and *Balaenoptera physalus* ($n = 9$). Geometric mean and range in brackets. (*B*) Plot of total organochlorines with BPMO activity in skin biopsies of *Stenella coeruleoalba* (1) and *Balaenoptera physalus* (2). Modified from Fossi et al. (*10*).

Table 1. Biological materials potentially obtainable by noninvasive techniques in vertebrates.

Biological materials	Mammals	Birds	Reptiles	Amphibians	Fish
Blood	+	+	+	+	+
Liver biopsy	+	+	+	+	+
Skin biopsy	+	–	–	–	–
Excreta	+	+	+	+	+
Milk	+	–	–	–	–
Hair	+	–	–	–	–
Antlers	+	–	–	–	–
Eggs	–	+	+	+	+
Feathers	–	+	–	–	–

+, Obtainable; –, not obtainable.

The potential use of several biological materials (blood, excreta, skin biopsy, liver biopsy, milk, hair, antlers, feathers, wings and eggs) suitable for biomarker and residue analysis is summarized in Table 1. The materials most easily obtainable by noninvasive techniques are reported in this table for each taxonomic group of vertebrates (fish, amphibians, reptiles, birds, mammals).

Blood is the tissue of choice for nondestructive biomarker/residue work. It is readily obtained with minimal risk and a wide range of biomarkers and residue levels can be measured in it. Its major limitations are the fact that residue levels may be momentarily high following ingestion of food containing especially high levels of contaminants, and that some biomarkers fluctuate more rapidly than in other organs.

Nondestructive Biomarker Techniques

The choice of biomarker or series of biomarkers to use in a biomonitoring program should be guided by the information to be obtained; in other words, one must consider whether the study is aimed at evaluating the overall environmental contamination or at a more specific evaluation of risk to the population of a specific species. Having decided which type of information is to be obtained, one should review the available methods and biological material of the species in question (Table 1).

Table 2 summarizes some of the biomarkers applicable with invasive and noninvasive methods, more commonly used in biomonitoring. For each biomarker, we give a succinct ID consisting of biological response and relationship to environmental pollution, invasive techniques and nondestructive techniques available, temporal occurrence, and reliability index.

Suitable nondestructive biomarkers include old and new generation biomarkers. The former (MFO activity, porphyrins, DNA adducts, esterases, etc.) were traditionally used with invasive methods but when modified can also be applied to material obtainable in a noninvasive way. The latter include methods conceived and standardized on noninvasive material (blood chemistry, vitamin A, micronuclei, etc.) normally used in clinical practice; to be applied in the field of ecotoxicology, they require validation.

Development and Validation of the Nondestructive Biomarker Approach

In the world of biomarker research, significant efforts have recently been made to identify potential new biomarkers. The characteristics of the "ideal" biomarker are as follows: measurement in readily available tissues or biological products obtainable in a noninvasive way; can be related the measurement to exposure and/or degree of harm to the organism; direct relation to the mechanism of action of the contaminants; highly sensitive techniques that require minimal quantities of sample and are easy to perform and cost effective; suitable for different species.

Many of these features may be found in the ordinary clinical tests used in medicine. Thoroughly tested in humans, these tech-

Table 2. Biomarkers for environmental monitoring: destructive and nondestructive use.

Biomarker	Biological response	Pollutant	Invasive techniques	Nondestructive techniques	Temporal occurrence	Reliability index
Esterases	Enzyme inhibition	OPs and CBs	Brain	Blood	Early	S,D,P
Porphyrins	Metabolic disorder	Toxic metals PHAHs	Liver	Bood Excreta Feathers?	Middle	S,D,P
MFO	Enzyme induction	PAHs, PHAHs	Liver	Skin Mucosa Blood?	Early	S,D
Blood chemistry	Various enzymes	Toxic metals PHAHs, OPs	—	Blood	Middle	S,D
Retinols	Retinol changes	PHAHs	Liver	Blood	Early	S
Thyroid function	Thyroid function alteration	PHAHs	Thyroid	Blood	Middle	S
ALAD	Inhibition	Toxic metals	—	Blood	Early	S,D,P
Immunotoxicology	Various	Toxic metals PAHs, PHAHs, OPs	Lymphatic cells	Blood	Middle/late	S
Hb adducts	Adducts	PAHs, PHAHs	—	Blood	Early	S,D
Stress proteins	Protein induction	Toxic metals, PHAHs	—	Blood	Early	S
DNA						
Strand breakage	Strand breaks	PAHs, PHAHs	Several tissues	Blood Skin	Early	S
Adducts	Adducts	PAHs, PHAHs	Several tissues	Blood Skin	Early	S,D,P
SCE	Chromosomes	PAHs, PHAHs	Several tissues	Blood	Middle/late	S,D,P

CBs, carbamates; OPs, organophosphates; PAHs, polynuclear aromatic hydrocarbons; PHAHs, polyhalogenated aromatic hydrocarbons; SCE, sister chromatid exchanges. Temporal occurrence: Early—hours to days; Middle—days to weeks/months; Late—weeks/months to years. Reliability index: (S, signal of potential problem; D, definitive indicator of type or class of pollutant; P, predictive indicator of a long-term adverse effect. Modified from Fossi et al. (*6*).

niques promise to be a rich source of new methods for use in environmental studies. One of the obstacles to this approach is the enormous interspecific variability even for the same biochemical process. Knowledge of species-specific basal levels of certain enzyme activities or metabolic processes is therefore essential if these techniques are to be extrapolated into the environmental field. Before it can be used in the field, a new biomarker requires much basic research into dose response relationships, and biological (age, sex, genetic stock, reproductive status, etc.) and environmental influences (temperature, salinity, light, etc.) on baseline values of responses.

One of the most important aspects in the validation of new nondestructive biomarkers is to investigate the relation between nondestructive and destructive biomarker responses in the tissues and target organs by laboratory experimentation. Only after a preliminary phase of this kind can a series of models, fo- application in field studies, be constructed.

An Example of Validation of Nondestructive Biomarkers: the Case of Esterases in Reptiles

In our department, we have recently been working on identifying the relationship between nondestructive and destructive biomarkers (particularly esterases) in different vertebrate species (*11–17*). The following experiment, performed in collaboration with the Department of Pedology and Geology of the University of La Laguna (Spain), is an example of this approach. The aim of this study (*17*) was to propose a bioindicator organism, the lizard *Gallotia galloti*, and a nondestructive biomarker, serum butyrylcholinesterase (BChE), for the assessment of the toxicologic impact of organophosphorus (OPs) insecticides in the Canary Islands (Spain). Laboratory and field studies were performed using the OP insecticide trichlorphon. In the laboratory study, experimental groups of *Gallotia galloti* were treated with 5 mg/kg, 50 mg/kg, and 100 mg/kg of trichlorphon, respectively. After 24 hr, the following enzyme activities were assayed: brain acetylcholinesterase (AChE) and serum butyrylcholinesterase (BChE). The recovery of BChE activity was monitored in two groups of lizards treated with 50 mg/kg and 100 mg/kg of trichlorphon, respectively, for 21 and 31 days after treatment. In the field study, BChE activity was detected in *Gallotia galloti* specimens, 24 and 48 hr after treatment of an experimental area with 2 kg/ha of Dipterex (80% Trichlorphon). Three main facts emerged from this preliminary experiment: *a*) *Gallotia galloti* has the features of an ideal bioindicator: high sensitivity to OPs and extremely slow recovery of serum BChE with respect to other vertebrate such as birds, normally used as sentinel species. This property enormously extends the practical possibilities of this biomarker of exposure, making it possible to detect the exposure to an OP compound not merely up to 48 to 72 hr after exposure as in birds, but for as long as 20 to 30 days. *b*) The results confirm the validity of the nondestructive biomarker BChE for detecting the exposure to OPs. A high correlation was found between the destructive biomarker brain AChE and the nondestructive biomarker serum BChE, 24 hr after treatment. The fact that the percentage inhibition of AChE can be predicted from the inhibition of the nondestructive biomarker BChE (Figure 3), within 24 hr of poisoning, transforms the significance of this index from biomarker of exposure to "surrogate biomarker of effect," greatly broadening its ecotoxicologic meaning. *c*) The results of the field study show the relative non toxicity of trichlorphon for non-target organisms at the average concentrations (2 kg/ha) used in agriculture. An exploratory approach based on biomarker strategy would enable the relative safety of the various OP compounds to be tested for non target organisms. Such information would be extremely useful for environmental control.

Conclusions

In conclusion the nondestructive biomarker approach appears to be a useful tool in ecotoxicology, particularly in the hazard assessment of endangered species of vertebrates. A large number of laboratory and field studies, such as the experiment presented in this paper, are needed to validate this methodologic approach. Effects observed in the field may suggest the further development of biomarkers in the laboratory; conversely, the development and validation of new techniques in the laboratory may provide the basis for a valuable field study method. Highly sensitive molecular biology techniques that require minimal quantities of sample need to be developed.

A concluding comment should be made about nondestructive biomarkers of geno-

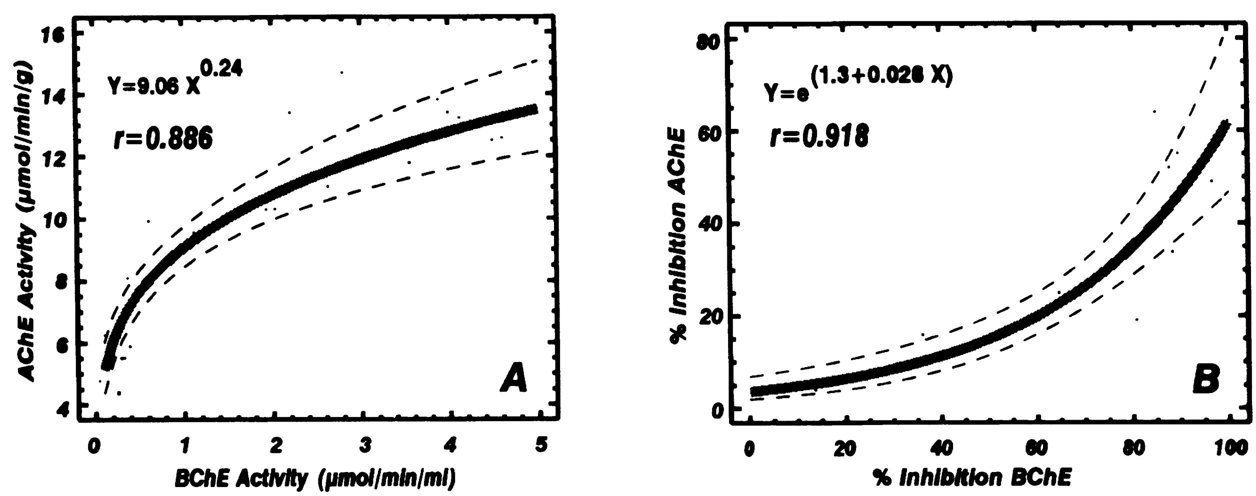

Figure 3. Correlation (*A*) between serum butyrylcholinesterase (BChE) activity and brain acetylcholinesterase (AChE) activity; and (*B*) between inhibition of serum butyrylcholinesterase (BChE) activity and inhibition of brain acetylcholinesterase (AChE) activity 24 hr after treatment of *Gallotia galloti* with 5 mg/kg, 50 mg/kg, and 100 mg/kg trichlorphon. Modified from Fossi et al. (*17*).

toxicity and particularly on the availability of DNA in blood for genotoxic testing. In a recent publication, Shugart (*18*) reported that the difficulty of obtaining a sufficient number of nucleated cells from mammalian blood may exclude the use of certain methodologies for nondestructive sampling. On the other hand, nonmammalian vertebrates (fishes, amphibians, reptiles, and birds) are more suitable candidates because they have nucleated red blood cells. In these species, a significant amount of high molecular weight DNA can be obtained from a small sample of blood without sacrificing the animal.

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